Exp Appl Acarol (2008) 46:105–117
DOI 10.1007/s10493-008-9160-z

Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on *Varroa* mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health

William G. Meikle · Guy Mercadier · Niels Holst · Vincent Girod

Received: 19 December 2007 / Accepted: 8 May 2008 / Published online: 28 May 2008

Abstract Bee colonies in southern France were treated with conidia (asexual spores) from two strains of *Beauveria bassiana*, an entomopathogenic fungus. One strain was commercial (GHA) and the other had been isolated from *Varroa* mites in the region (Bb05002). Objectives were to evaluate treatment effect on colony weight, adult bee mass, capped brood, and on *Varroa* fall onto sticky boards. Treatments included conidia formulated with either carnauba or candelilla wax powder, candelilla wax powder alone, or control; in two treatment groups formulation was applied a second time after one week. Treatment did not affect colony health. Colonies treated twice with Bb05002 conidia and carnauba wax powder had significantly higher mite fall compared to colonies treated with blank candelilla wax powder. The proportion of fallen mites that were infected in both conidia treatments was higher than controls for 18 days after the second treatment. The number of fungal propagules on the bees themselves remained elevated for about 14 days after the second treatment. These results were compared to published results from previous experiments with regard to infection duration.

Keywords *Apis mellifera* · *Varroa destructor* · *Beauveria bassiana* · Biopesticide · Formulation

W. G. Meikle (✉) · G. Mercadier
European Biological Control Laboratory, USDA–ARS, Campus International de Baillarguet, CS 90013 Montferrier sur Lez, St. Gely du Fesc, 34988 Montpellier, France
e-mail: wmeikle@ars-ebcl.org

N. Holst
Faculty of Agricultural Sciences, Department of Integrated Pest Management, University of Aarhus, Flakkebjerg, 4200 Slagelse, Denmark

V. Girod
ADAPRO LR-CRALR, Maison des Agriculteurs, Mas de Saporta CS 30012, Lattes, 34875 Montpellier, France

Introduction

Varroa destructor Anderson and Trueman, one of the most serious pests of honeybees (*Apis mellifera* L.) (Hymenoptera: Apidae) (Chandler et al. 2001; Martin 1998), weaken larvae and adults by feeding on haemolymph, transmitting diseases, and inducing deformities (Chandler et al. 2001; Martin 2001). *Varroa* infestations have been largely responsible for the almost complete elimination of feral colonies in the U.S. (Rinderer et al. 2001). The use of entomopathogenic fungi has been considered a promising alternative to chemical miticides (Chandler et al. 2001), and fungal isolates of several species have been tested (James et al. 2006; Kanga et al. 2003, 2006; Meikle et al. 2006, 2007, 2008; Shaw et al. 2002). Meikle et al. (2006) reported the discovery of several isolates of *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycota: Hyphomycetes) from *Varroa* mites collected from honeybee colonies in southern France. Collecting fungal isolates from either the target environment and/or even the target pests themselves is intended to increase the probability of finding the best adapted isolates. For example, an isolate of *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycota: Hyphomycetes), collected from cadavers of *Ornithacris cavroisi* (Finot) (Orthoptera: Acrididae) in Niger, was found in tests to be well-adapted to the high temperatures and low humidities of the region and was subsequently incorporated into a biopesticide against the desert locust *Schistocerca gregaria* Forskål (Orthoptera: Acrididae) (Cherry et al. 1999; Lomer et al. 1997). The high temperatures and humidities of beehives, among other factors, can present similar challenges to entomopathogenic fungi.

Meikle et al. (2007, 2008) used a *B. bassiana* isolate, collected from *Varroa*, in field experiments where it significantly increased *Varroa* fall and was found on the surfaces of bees and infecting fallen *Varroa* mites for at least a week after a single treatment. The use of an entomopathogenic fungus in an insect colony involves some risk. *Beauveria bassiana* is known to have a broad host range (Tanada and Kaya 1993) and Meikle et al. (2006) reported *B. bassiana* infections of bee pupae that had been exposed to treated *Varroa* mites in laboratory bioassays. However, Jaronski et al. (2004) and Meikle et al. (2008) found that a single application of *B. bassiana* did not have measurable negative impact on colony health or survivorship. Meikle et al. (2008) observed that while a single application significantly increased mite fall, it did not have sufficient negative impact on *Varroa* densities to be considered as a complete control strategy.

Entomopathogenic fungi are usually applied in the form of conidia, which are asexual, non-motile spores (Burges 1998), and the conidia are often combined with other materials in order to stabilize the conidia during storage, facilitate application, protect the conidia, and enhance conidia activity (Jones and Burges 1998). Carnauba wax, obtained from *Copernicia cerifera* or *C. prunifera*, is hydrophobic and inert, with no nutritive value for the conidia, and is permitted as a food additive (U.S. Code of Federal Regulations, Title 21, part 184, Sect. 1978) so it poses no honey contamination problems in the U.S. Formulation with carnauba wax has been tested in previous studies and no negative effects have been observed with respect to honeybee colony health (Meikle et al. 2007, 2008). Meikle et al. (2008) also formulated conidia with wheat flour, which is different from wax powders because it can be used as a food source for germinating conidia and is therefore not an inert ingredient (Burges 1998). Meikle et al. (2008) found that the impact of the wheat flour formulation on mite fall was significantly less than conidia combined with carnauba wax powder. Treating hives with a powder, such as powdered sugar or pollen, is known to provoke mite fall (Fakhimzadeh 2001; Macedo et al. 2002), but 99% of the effect occurs within 18 h (Fakhimzadeh 2001).

The main objective in this study was to evaluate the impact of two successive applications of conidia of entomopathogenic fungi on colony health and on *Varroa* mite fall. Two isolates of *B. bassiana* were used: isolate 05002 (NRRL 30976) and strain GHA (Laverlam International, Butte MT; Technical Grade Active Ingredient lot number 03-04-1C/1D). Two kinds of wax powder were also compared as formulation ingredients: carnauba wax, as used in previous experiments, and candelilla wax, which is obtained from *Euphorbia antisyphilitica* and *Pedilanthus pavonis* and is also safe as a human food ingredient (U.S. Code of Federal Regulations, Title 21, part 184, Sect. 1976). Here we measured colony growth rates per week, total adult bee weight and the amounts of sealed brood and honey. The use of growth rates, which are independent of colony size, was intended to facilitate comparison of these results with other studies. *Varroa* mite fall and the proportion infected mites were measured as in Meikle et al. (2007).

Materials and methods

Preparation of formulation

Cultures of *B. bassiana* isolate Bb05002 were grown on Sabouraud dextrose agar with yeast (SDAY) (Goettel and Inglis 1997) for a minimum of 15 days. Conidia were harvested by scraping the surface of the cultures onto glass petri dishes with a metal spatula, and placing the petri dishes in a crystallizing dish containing silica gel for 20–24 h at room temperature for drying.

Four formulations were prepared: carnauba wax powder and conidia from *B. bassiana* isolate 05002 ("Bb05002 + carnauba"); carnauba wax powder and conidia from *B. bassiana* isolate GHA ("BbGHA + carnauba"), candelilla wax powder and 05002 conidia ("Bb05002 + candelilla") and candelilla wax powder alone ("candelilla alone"). Since carnauba wax powder alone had been tested in previous experiments (see Meikle et al. 2007, 2008), it was not tested here. All formulations were prepared on 8 May. The per colony dose of Bb05002 + carnauba and BbGHA + carnauba consisted of 1.0 g conidia of the respective isolate mixed with 9.0 g carnauba wax powder (Strahl & Pitsch Inc., West Babylon, NY, USA) and 0.05 g hydrated silica (Hi-Sil-233, Pittsburgh Plate Glass, Pittsburgh, PA, USA) as a flow agent. The per colony dose of Bb05002 + candelilla consisted of 1.0 g conidia of isolate 05002 mixed with 9.0 g candelilla wax powder (Strahl & Pitsch Inc., West Babylon, NY, USA) and 0.05 g hydrated silica. The per colony dose of candelilla alone consisted of 9.0 g candelilla wax powder and 0.05 g silica. All formulations were mixed using a food processor (Valentin Mini Chopper, SEB, Dijon, France). The density of colony-forming units (cfu) per g formulation was determined at the time of colony treatment by plating three sub-samples of the formulation diluted in distilled water and Tween 80 (Merck, Munich, Germany) onto potato-dextrose agar, and counting the number of colonies 96 h after plating. Formulated and unformulated conidia were stored in a refrigerator at 4°C.

Field experiment

In April 2007, 26 honeybee colonies were selected for the field experiment. The colonies were part of an apiary of 52 colonies near Lattes, in southern France. The bee colonies were kept in painted, 10-frame, wooden Langstroth brood boxes (56 l capacity) with telescoping lids and with screens underneath the frames and queen excluders on top of the

brood box. On 17 April one sticky board (31 × 42 cm, Mann Lake Ltd, Hackensack, MN, USA) was placed under each colony. The boards were replaced on 24 April and every 3–4 days thereafter with fresh boards. All mites adhering to the used boards were counted, and 40-mite samples were taken from each board and plated on water agar (6.0 g/l) with chloramphenicol (0.4 g/l). If a board had 40 or fewer mites, all mites were plated. Plated mite samples were incubated at 23°C, examined for sporulation after 15 days, and the proportion of sporulating cadavers was calculated (hereafter referred to as the "proportion infected mites"). Temperature loggers (Thermachron iButton, Dallas Semiconductor, Sunnyvale, CA, USA) were placed in seven hives at the center of the queen excluder on the top of the brood box to record internal temperature hourly starting the day of treatment, and another logger was placed nearby in the shade to record ambient temperature.

On 2 May and at seven-day intervals until 8 June (after which hives were moved and the experiment stopped) each hive was weighed using a portable electronic balance with a precision of 50 g (OHaus Corporation model Champ CQ100L, Pine Brook, NJ, USA). On 9 May, and again on 27 June, each hive was opened and each hive part (i.e. brood box, lids, colony base, and frames after shaking them free of bees) was weighed using a smaller portable electronic balance with a precision of 1 g (Kern & Sohn model 12 K 1 N, Balingen, Germany). Digital photographs were taken of each side of each frame using a 3.3 megapixel camera (Nikon Coolpix 990, Tokyo, Japan). The hive was then reassembled, and one super containing nine frames with wax foundation was weighed and placed on top of each colony. On 27 June, the super was also weighed.

Five colonies were selected for each treatment group except the untreated control, which had 6. Colonies were randomly assigned treatments, but treatments were occasionally re-assigned to distribute treatments evenly. Colonies in the Bb05002 + carnauba, BbGHA + carnauba and candelilla alone treatments were treated on 10 and 17 May. Colonies in the 05002 + candelilla treatment were treated only once, on 10 May. For each colony treatment, a plastic laboratory wash bottle (Nalge Nunc International, Rochester, NY) was filled with a single dose of preparation, the hive lid and super removed, the formulation blown between all brood box frames by squeezing the wash bottle, and the super and lid replaced.

To calculate colony and adult bee weight, hive weight was divided into a "non-colony" part, consisting of the hive pieces, e.g., brood box, lids, super, hive base, and 10 empty frames with foundation comb, and the "colony" part, consisting of the adult bees, brood, honey, pollen and wax (other than foundation comb). Adult bee weight was calculated as the difference between the sum of the weights of all the hive parts and the observed hive weight. The non-colony weight was calculated as the total weight of all the hive parts except brood box frames, plus the weight of 10 empty frames, or about 2.87 kg (Meikle et al. 2008). Colony weight was calculated by subtracting the non-colony component from the total hive weight. The area of sealed brood and sealed honey per frame was estimated from the photographs using ArcView 3.0 (Environmental Systems Research Institute, Redlands, CA, USA). Brood areas were inspected closely for any signs of fungal infection. Colony entrances were inspected for unusually large numbers of dead bees.

On 2, 9, 11, 15, 18, 22 and 25 May, and on 1 and 8 June, samples of approximately 15 bees were collected from within each hive into plastic boxes in the field, the boxes placed in a cooler with ice packs, and the boxes transferred to a freezer. Two subsamples of five bees each were removed from bags of three colonies per treatment (the same colonies were always used) for a total of 30 subsamples. Each subsample was placed in a 50 ml plastic centrifuge tube and vortexed for 3 min. in 10 ml of a 0.1% aqueous solution of Tween 80. Aliquots of 20 and 100 μl of the resulting suspension from each subsample were spread

onto each of three petri dishes containing potato dextrose agar with chloramphenicol (0.4 g/l); thus six plates for each subsample. The dishes were incubated for at least 14 days at 23°C, and the number of *B. bassiana* cfu were counted in the plates with 20 µl of solution; when cfu densities became low, cfu were counted on the 100-µl plates.

Statistical analysis

Data were analyzed using SAS (SAS Institute, Inc., Cary NC, USA) software. Repeated measure analyses of variance were conducted for a linear mixed model using PROC MIXED of SAS (Littell et al. 1996) with either mite fall (log transformed), or the proportion infected mites (arcsine square-root transformed) as the response variable and with three fixed effects: treatment, date and their interaction ($\alpha = 0.05$). The covariance matrix of both response variables was inspected for patterns and residual plots were assessed visually for variance homogeneity. Colony number was incorporated as a random effect. The degrees of freedom were calculated using the Satterthwaite method. Analyses were designed to maximize the degrees of freedom for detection of differences among treatments. Insignificant main effects were excluded from the model but if the interaction was significant both main factors were retained. Post hoc contrasts of the least squares means differences were conducted for all significant factors, using Bonferroni adjustment for the t-value probability. Because excess formulation on sticky boards immediately after treatment may cause spurious infection data, the 1st sample after treatment was excluded from analysis. Daily intrinsic natural rates of increase, *r*, were calculated for colony weight by dividing the observed value by the value for the previous sampling occasion and then dividing the logarithm of that ratio by the number of days between the two measurements (7). The *r* values for total adult bee weight and brood surface area were calculated in a similar manner by dividing the post treatment value (5 June) by the pretreatment value (9 May), and then dividing the logarithm of that ratio by the number of days between these two dates (27).

Results

Cfu density at time of treatment was 3.70×10^{10} cfu/g for the Bb05002 + carnauba formulation, 1.79×10^{10} cfu/g for the Bb05002 + candelilla formulation and 1.72×10^{10} cfu/g for the BbGHA + carnauba formulation. Average temperature at the top interior of the brood boxes was 30.0°C (average minimum = 21.4°C and average maximum = 37.8°C).

In the analysis of colony weight r values, treatment ($F_{4,116} = 4.72$, $P = 0.0014$), and date ($F_{4,116} = 25.29$, $P < 0.0001$) were significant factors, but their interaction was not ($P = 0.870$) (Fig. 1). Colony growth in the candelilla alone treatment was significantly greater than the Bb05002 + carnauba treatment ($t_{116} = 3.26$, $P = 0.0144$) and the control treatment ($t_{116} = 4.10$, $P = 0.0008$). Starting the day before treatment, the average total weight gain (s.e.) for colonies treated with Bb05002 + carnauba was 4.8 kg (0.8); for those treated with BbGHA + carnauba was 6.8 kg (2.4); for those treated with Bb05002 + candelilla 6.0 kg (2.3); for those treated with candelilla alone was 11.6 kg (1.8); and for the control colonies was 2.7 kg (4.8). One colony in the candelilla alone group gained 17.8 kg, exceeding by 5.3 kg the next highest colony weight gain; removing that datum reduced the treatment average to 10.0 kg. Treatment with conidia did not significantly affect the changes in surface areas of sealed brood ($P = 0.905$); overall, brood

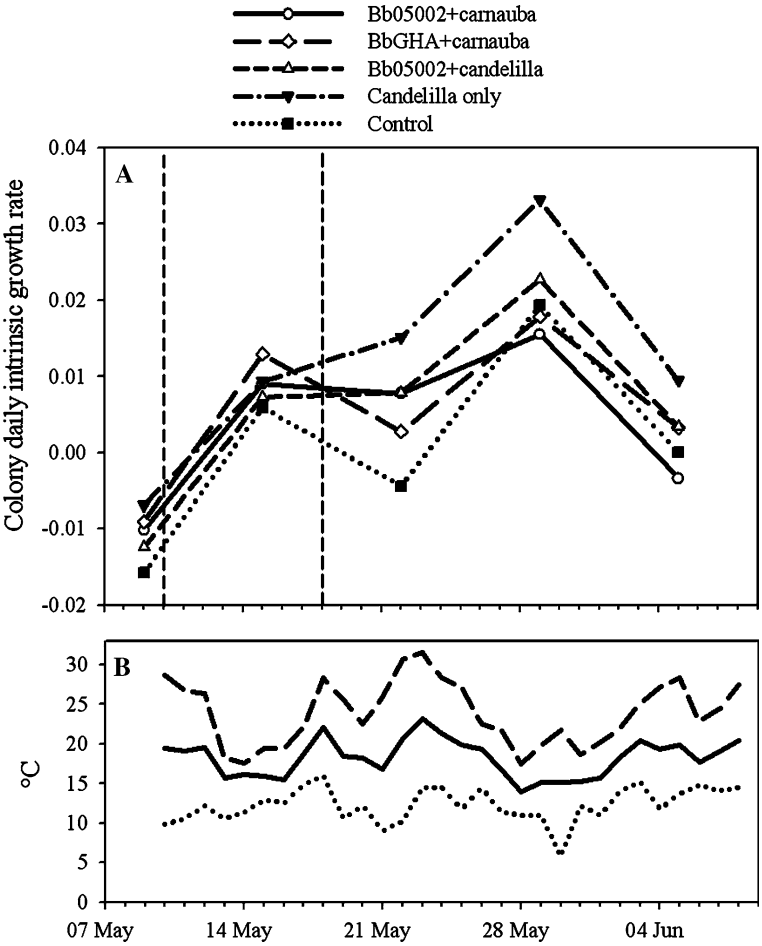

Fig. 1 Daily intrinsic growth for bee colonies treated with *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder (2 treatments), *B. bassiana* isolate GHA conidia + carnauba wax powder (2 treatments), isolate 05002 conidia + candelilla wax powder (1 treatment), candelilla wax powder alone (2 treatments), and untreated control in an experiment conducted in May 2007 near Lattes in southern France. (**a**) Average daily intrinsic rates of increase, r, of bee colonies; (**b**) Daily minimum (dotted line), maximum (dashed line) and average temperature (solid line) (°C). Vertical dashed line shows treatment dates

surface area declined on average by about 1.3% during the course of the experiment. Brood loss was 6.2% (6.0) in the Bb05002 + carnauba treatment, 1.8% (2.2) in the BbGHA + carnauba treatment, 0.6% (0.5) in the candelilla alone treatment and 2.2% (4.0) in the control treatment while hives in the Bb05002 + candelilla treatment gained an average of 2.1% (2.5). No infected brood were observed in any photographs. Treatment did not significantly affect total adult weights ($P = 0.460$); average total adult weights increased by 0.32 kg (0.62) in the Bb05002 + carnauba treatment, by 0.92 kg (0.41) in the BbGHA + carnauba treatment, by 0.24 kg (0.43) in the Bb05002 + candelilla treatment, and by 1.67 kg (0.21) in the candelilla alone treatment; hives in the control treatment lost an average of 0.27 kg (0.81).

In the analysis of mite fall, treatment was significant ($F_{2,245} = 4.94$, $P = 0.0008$) but neither date ($P = 0.999$) nor treatment × date ($P = 0.999$) were (Fig. 2). Post hoc contrasts showed that mite fall in hives treated with 05002 + carnauba was significantly higher than in hives treated with candelilla powder alone ($t_{245} = 4.35$, $P = 0.0002$). No other contrasts were significant. Treatment ($F_{4,160} = 62.45$, $P < 0.0001$), date ($F_{7,160} = 7.50$, $P < 0.0001$) and treatment x date ($F_{28,160} = 1.77$, $P = 0.0150$) were all significant factors in explaining the proportion of infected mites. In post hoc contrasts treatment was a significant factor (P always <0.0001) for all dates through 1 June. Treatment was not significant for mites collected on 5 June, but it was again significant ($P = 0.0027$) for mites collected on 8 June.

The proportion of infected mites in the treatments Bb05002 + carnauba and BbGHA + carnauba were significantly different from zero for all days (P always <0.0001); the same result held for the treatment Bb05002 + candelilla except that the probability varied between <0.0001 and 0.0339 (Fig. 3). Among the hives not treated with spores, the only occasion on which the proportion of infected mites rose to significance occurred the candelilla alone treatment for mites collected on 5 June. At least one infected mite was found in three of the experimental hives during the three weeks before application. Infected mites were also found in hives not treated with conidia, probably due to bee drift or robbing. The infection rate after the second treatment appeared to remain

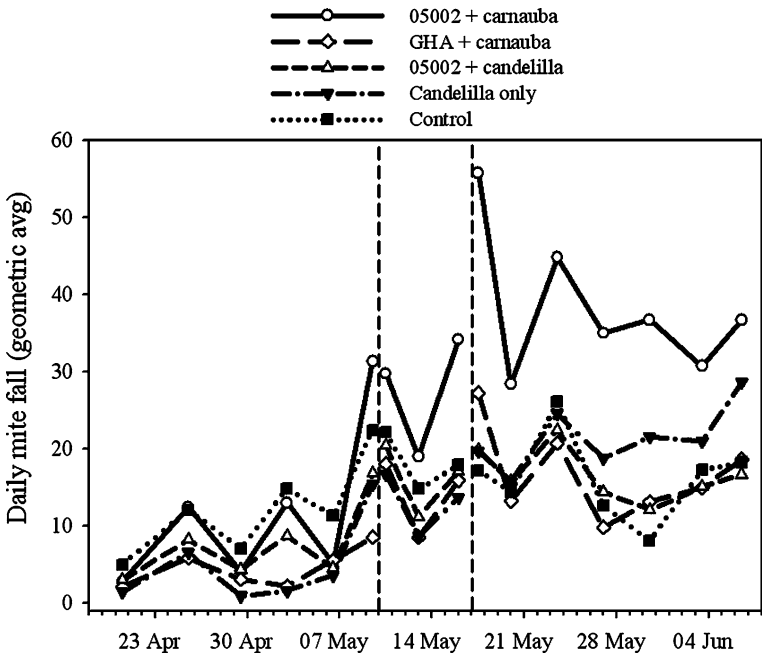

Fig. 2 Daily fall (geometric average of x + 0.01) of mites onto sticky boards placed under bee colonies treated with *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder (2 treatments), *B. bassiana* isolate GHA conidia + carnauba wax powder (2 treatments), isolate 05002 conidia + candelilla wax powder (1 treatment), candelilla wax powder alone (2 treatments), and untreated control in an experiment conducted in May 2007 near Lattes in southern France. Vertical dashed line shows treatment dates

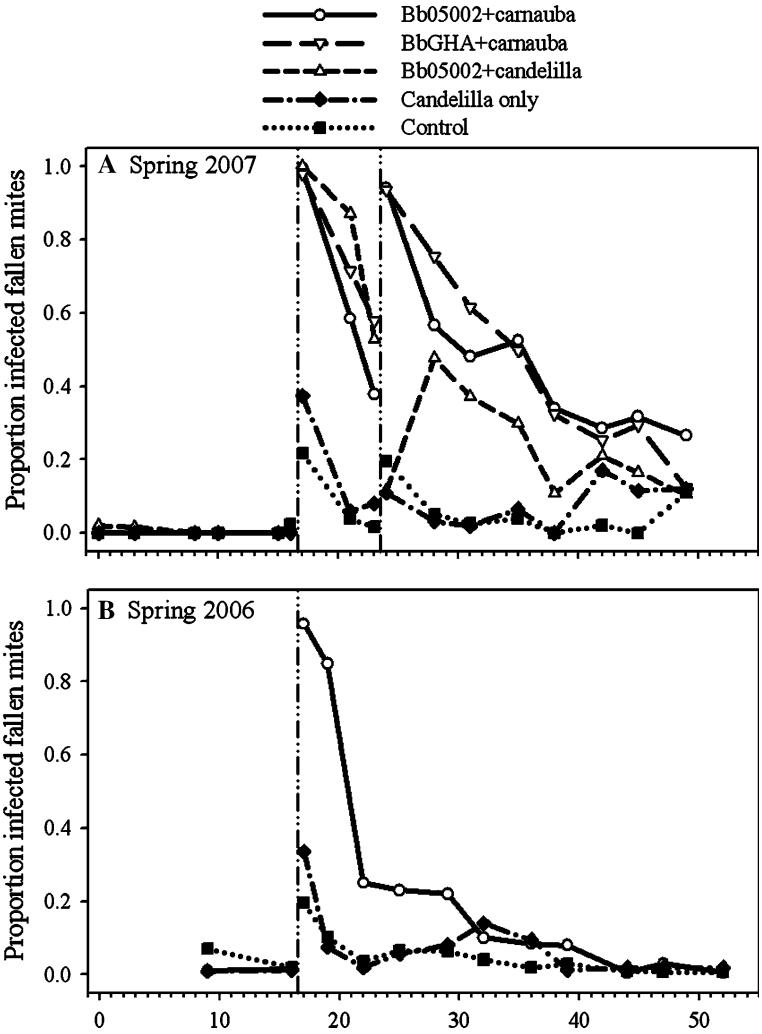

Fig. 3 Proportion of fallen mites that were found to be infected by *B. bassiana* among bee colonies in two experiments near Lattes in southern France. (**a**) colonies treated in Spring, 2007, with *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder (2 treatments), *B. bassiana* isolate GHA conidia + carnauba wax powder (2 treatments), isolate 05002 conidia + candelilla wax powder (1 treatment), candelilla wax powder alone (2 treatments), and untreated control. (**b**) colonies treated in Spring 2006, with one treatment of either *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder or carnauba wax powder alone, and untreated control. Data from Meikle et al. (2007). Vertical dashed line shows treatment days

elevated for a longer time than in hives treated with a single application of Bb05002 + carnauba in Spring, 2006.

The analysis of cfu density was conducted in the same manner as for the proportion infected mites. No *B. bassiana* cfu were observed in any bee samples before application. Treatment ($F_{4,70} = 42.99$, $P < 0.0001$), date ($F_{6,70} = 43.11$, $P < 0.0001$) and treatment × date ($F_{24,70} = 5.59$, $P < 0.0001$) were significant overall (Fig. 4). After

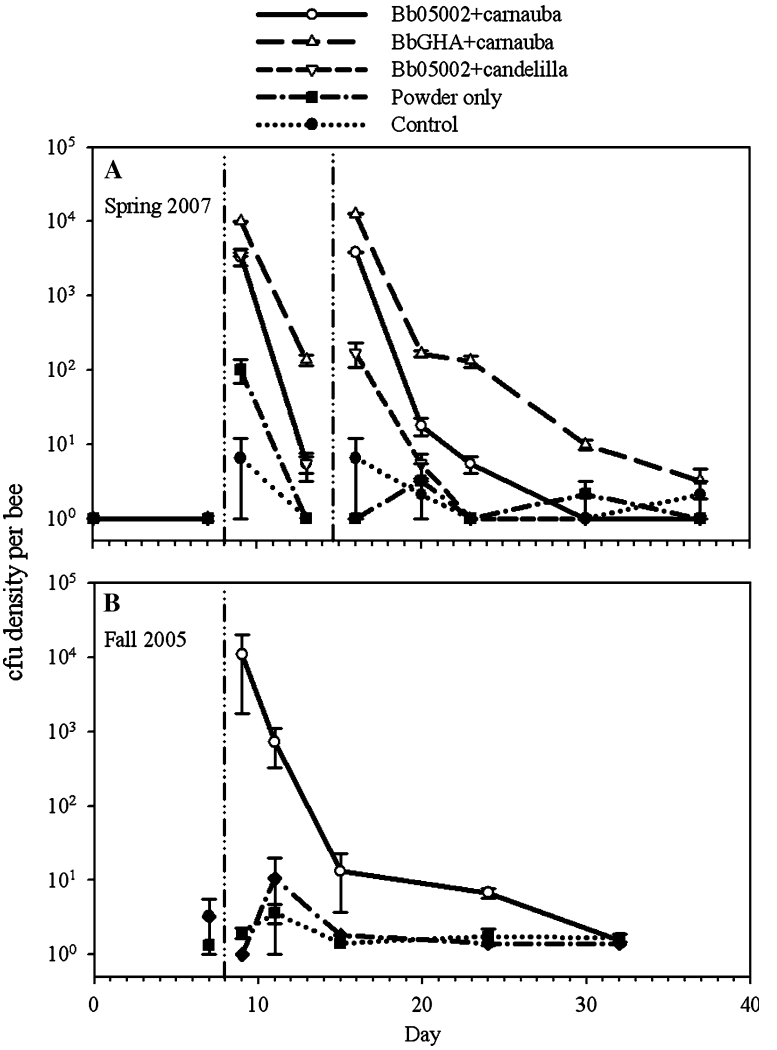

Fig. 4 Density of colony-forming units (cfus) per bee in two experiments in southern France (log scale). (**a**) colonies treated in Spring, 2007, with *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder (2 treatments), *B. bassiana* isolate GHA conidia + carnauba wax powder (2 treatments), isolate 05002 conidia + candelilla wax powder (1 treatment), candelilla wax powder alone (2 treatments), and untreated control. (**b**) colonies treated in Fall 2005, with one treatment of either *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder or carnauba wax powder alone, and untreated control. Vertical dashed line shows treatment days. Data from Meikle et al. (2007)

application, treatment was a significant factor on all days (P varied from <0.0001 to 0.0245) until the samples collected on 1 June, after which treatment was no longer significant. The day after the 1st application, 11 May, cfu densities in the Bb05002 + carnauba ($t_{70} = 10.54$, $P < 0.0001$), BbGHA + carnauba ($t_{70} = 12.81$, $P < 0.0001$) and Bb05002 + candelilla ($t_{70} = 11.33$, $P < 0.0001$) were all significantly different from 0 but in the next sample four days later only the cfu density in the BbGHA + carnauba treatment was significant. In the samples collected just after the 2nd

application on 18 May, all three treatments using *B. bassiana* were significant but by the next sample only the BbGHA + carnauba treatment was significant; it remained so until the last sample on 8 June. The decline in cfu density per bee was very similar to that observed in Fall, 2005, in hives treated with a single application of Bb05002 + carnauba.

Discussion

The goals of this study were to evaluate the effects of two applications of *B. bassiana* conidia, with two different strains and formulated with two kinds of wax powder, on bee colony health and on *Varroa* mite fall. The experiment was conducted in the spring, when brood densities and foraging activity were high and the sensitivity of hives to a perturbation likewise high. While hives are generally not treated against *Varroa* in the spring in southern France (Meikle et al. 2008), this period was chosen because a negative treatment effect would be expected to have a quantitatively greater impact on adult bee and brood populations and/or colony food stores and weight gain. We found no negative effect of application of entomopathogenic fungi on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood, and colony survivorship. Colony growth among all groups was lowest immediately after application, but this was likely due to food consumption prior to a nectar flow. Colony growth increased among all groups thereafter. No treatment differences were observed in either total adult weight change or changes in the amounts of sealed brood or honey. Little impact of *B. bassiana* application in beehives has been observed in similar studies elsewhere (Jaronski et al. 2004; Meikle et al. 2008). While some workers (e.g. Kanga et al. 2003) have collected and plated dead adult bees in an effort to measure bee mortality due to mycosis, this was not done here because it was felt the data would be difficult to interpret properly. As observed here, and by Meikle et al. (2007) and Kanga (2003), adult bees retain *B. bassiana* cfus on their bodies for days and even weeks after application. Since the length of time between a given bee's death, its ejection from the hive and its collection by the researcher is unknown, the presence of sporulation on these cadavers, even with surface sterilization, would not reliably indicate whether the fungus killed the bee.

Treatment was a significant factor in explaining average daily mite fall, and colonies treated with Bb05002 + carnauba had significantly higher mite fall than those treated with candelilla powder alone. However, average mite falls in treated hives were always higher than both controls and candelilla powder alone within the time frame of this study so fungal treatment did not lower mite densities as they were measured here. Mite fall in hives treated with isolate GHA was not significantly different than the control hives. These results should not be considered an indication that GHA could not be effective in this context because the number of replicates per treatment was too low for a definitive answer. Davidson et al. (2003) found that BbGHA was highly virulent against *Varroa* mites in lab bioassays. Further work with different isolates is needed to determine the role that isolate characteristics play in the field control of *Varroa* mites. The powder + conidia formulation apparently distributed well in hives; the cfu densities per bee found here were similar to those found in an earlier experiment by Meikle et al. (2007) as well as by Kanga et al. (2003) who used at least 40 g spores per hive.

While large numbers of fallen mites were infected with *B. bassiana*, they clearly did not all die due to infection. As observed by Meikle et al. (2007, 2008) a mite with viable conidia on its cuticle may fall for other reasons, and in the 3–4 days between board replacement conidia could germinate on the dead or dying mite, resulting in a false positive

because the fungus did not cause the mite to drop. *Beauveria bassiana* conidia grow readily on cadavers (Tanada and Kaya 1993), and surface sterilization of the mites would reliably remove only some of those false positives - those less than 1–2 days old (the time needed by the fungus to establish itself within a mite). Given the low probability of false negatives (in which a mite dies from fungal infection but the cadaver does not sporulate), the proportion of infected mites should be considered upper-bound estimates of the true percentage of infection, and not necessarily related to how well the treatment works against *Varroa*. However, these data can be considered indicators for the presence of viable *B. bassiana* propagules in the hive.

Conidia formulated with candelilla wax was not measurably different, in terms of cfu per bee or proportion infected mites, from those formulated with carnauba wax. This supports the hypothesis that properties the two waxes have in common, such as being hydrophobic and lipophilic, are those properties that are important as formulation ingredients. The group treated once with Bb05002 + candelilla wax powder did distinguish itself in one important regard: although those hives were only treated once, the proportion of infected mites increased significantly in a manner very similar to the hives in the two groups that were treated twice with fungal formulation. Of the 26 hives in the experiment, only seven hives increased in terms of proportion infected mites between 22 and 25 May, that is, between five and eight days after hives in the Bb05002 + carnauba and BbGHA + carnauba groups were treated a second time. Of those seven hives, one was in the BbGHA + carnauba group (increase of 9%), one in the control group (increase of 7%) and the remaining five comprised all the hives in the Bb05002 + candelilla group, with an average increase of 36% (s.e. = 0.06) and a range of 25–59%. The likelihood that the five hives with by far the largest increases in infection rate would randomly turn up in the same treatment group is low: <0.001. That bees with *B. bassiana* propagules on their bodies, as well as infected mites, were found in that treatment group indicate that those bees must have visited treated hives and returned to their colonies. While this could result from bees robbing treated hives, or bee drift, why this would occur among all the hives of one treatment and essentially none of the hives in other treatments is curious.

Spore viability over time was not directly measured in the hives but it is likely that *B. bassiana* can survive there. Aerial conidia are known to tolerate high temperatures (Burges 1998). Although brood mass temperatures range from 33–36°C (Southwick 1991; Winston 1987), temperatures in broodless areas tend to be lower (Simpson 1961). The average temperature on top of the queen excluder between the brood box and the super in this experiment was 30.0°C. Meikle et al. (2008), in an experiment conducted in the same location the previous year but later in the spring (thus with higher ambient temperatures), recorded temperatures at the bottom of the brood box from 30–32°C. Temperatures in this range present little problem for either survivorship or germination of *B. bassiana* conidia. Davidson et al. (2003) observed growth in all seven of their *B. bassiana* isolates at 30°C and in five of those isolates at 35°C, and Fargues et al. (1992) observed growth in all three isolates at 32°C and one of those at 35°C. The isolate used here germinated at 34°C (Meikle et al. 2008), so apparently conditions in much of the hive would not have prevented conidium survivorship or germination. Using a simulation model of conidium longevity (Meikle et al. 2003) based on the relationship between r.h. and conidium moisture content described by Hong et al. (2002), at 35°C the half lives of the eight *B. bassiana* isolates described in Hong et al. (2001) were estimated to be 43–135 d at 40% r.h. and 4–13 d at 70% r.h.

The proportion infected mites in the colonies treated with conidia was significantly higher than in controls for about 18 days after application. Meikle et al. (2008) reported

significantly higher infection rates for less than a week after a single application, while Meikle et al. (2007) observed higher infection more than a month after single applications in two experiments conducted in the fall. Infection half life would have been affected by colony dynamics, weather conditions, and their interaction. A large emergence of young bees would dilute cfu density among bees and mites and thus shorten infection half life as it was measured here.

In these experiments no impact on colony health was observed after two successive applications of formulation containing *B. bassiana* conidia. Two applications of the biopesticide increased mite fall relative to the blank powder treatment but did not reduce mite fall during the four weeks between the first application and the end of the experiment treatment. Future experiments will include more replicates per treatment, to better distinguish any treatment effects, and other measures of mite density, such as number of mites per adult bee, in addition to mite fall onto sticky boards. The results thus far are encouraging, but further work is clearly needed concerning conidia dosage, number of applications, and the ecology of entomopathogenic fungi within the beehive under ambient conditions and colony age structures.

Acknowledgments The authors would like to thank F. Annas for help in the field; S.T. Jaronski for supplying the GHA material; K. Hoelmer and W.A. Jones for constructive comments on the manuscript, and three anonymous reviewers for help in improving the manuscript.

References

Burges HD (1998) Chapter 4. Formulation of mycoinsecticides. In: Burges HD (ed) Formulation of microbial biopesticides: Beneficial microorganisms, nematodes and seed treatments. Kluwer Academic Publishers, Dordrecht, The Netherlands

Chandler D, Sunderland KD, Ball BV, Davidson G (2001) Prospective biological control agents for *Varroa destructor* n. sp., an important pest of the European honey bee, *Apis mellifera*. Biocontr Sci Technol 11:429–448

Cherry A, Jenkins N, Heviefo G, Bateman RP, Lomer C (1999) A West African pilot scale production plant for aerial conidia of *Metarhizium* sp. for use as a mycoinsecticide against locusts and grasshoppers. Biocontr Sci Technol 9:35–51

Davidson G, Phelps K, Sunderland KD, Pell JK, Ball BV, Shaw KE, Chandler D (2003) Study of temperature-growth interactions of entomopathogenic fungi with potential for control of *Varroa destructor* (Acari: Mesostigmata) using a nonlinear model of poikilotherm development. J Appl Microbiol 94:816–825

Fakhimzadeh K (2001) Effectiveness of confectioner sugar dusting to knock down *Varroa destructor* from adult honey bees in laboratory trials. Apidologie 32:139–148

Fargues J, Maniania NK, Delmas JC, Smits N (1992) Influence de la temperature sur la croissance in vitro d'hyphomycetes entomopathogenes. Agronomie 12:557–564

Goettel MS, Inglis GD (1997) Fungi: Hyphomycetes. In: Lacey L (ed) Manual of techniques in insect pathology. Academic Press, San Diego, CA, USA

Hong TD, Ellis RH, Gunn J, Moore D (2002) Relative humidity, temperature, and the equilibrium moisture content of conidia of *Beauveria bassiana* (Balsamo) Vuillemin: a quantitative approach. J Stored Prod Res 38:33–41

Hong TD, Gunn J, Ellis RH, Jenkins NE, Moore D (2001) The effect of storage environment on the longevity of conidia of *B. bassiana*. Mycol Res 105:597–602

James RR, Hayes G, Leland JE (2006) Field trials on the microbial control of *Varroa* with the fungus *Metarhizium anisopliae*. Am Bee J 146:968–972

Jaronski ST, Goettel MA, Lomer C (2004) Chapter 12. Regulatory requirements for ecotoxicological assessments of microbial insecticides - how relevant are they? In: Hokkanen H, Hajek A (eds) Environmental impacts of microbial insecticides: Need and methods for risk assessment. Kluwer Academic Publishers, Dordrecht, The Netherlands

Jones KA, Burges HD (1998) Chapter 2. Technology of formulation and application. In: Burges HD (ed) Formulation of microbial biopesticides: Beneficial microorganisms, nematodes and seed treatments. Kluwer Academic Publishers, Dordrecht, The Netherlands

Kanga LHB, Jones WA, James RR (2003) Field trials using the fungal pathogen, *Metarhizium anisopliae* (Deuteromycetes: Hyphomycetes) to control the ectoparasitic mite, *Varroa destructor* (Acari: Varroidae) in honey bee, *Apis mellifera* (Hymenoptera: Apidae) colonies. J Econ Entomol 96:1091–1099

Kanga LHB, Jones WA, Garcia C (2006) Efficacy of strips coated with *Metarhizium anisopliae* for control of *Varroa destructor* (Acari: Varroidae) in honey bee colonies in Texas and Florida. Exp Appl Acarol 40:249–258

Littell RC, Milliken GA, Stroup WW, Wolfinger RD (1996) SAS system for mixed models. SAS Institute, Cary, NC, USA

Lomer CJ, Prior C, Kooyman C (1997) Development of *Metarhizium* spp. for the control of locusts and grasshoppers. Mem Ent Soc Canada 171:265–286

Macedo PA, Wu J, Ellis MD (2002) Using inert dusts to detect and assess *Varroa* infestations in honey bee colonies. J Apic Res 41:3–7

Martin S (1998) A population model for the ectoparasitic mite *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies. Ecol Model 109:267–281

Martin SJ (2001) The role of *Varroa* and viral pathogens in the collapse of honey bee colonies: a modelling approach. J Appl Ecol 38:1082–1093

Meikle WG, Jaronski S, Mercadier G, Quimby PC (2003) A distributed delay routine-based simulation model of *Beauveria bassiana* conidial stability in response to environmental stressors. Biocontrol 48:561–578

Meikle WG, Mercadier G, Girod V, Derouané F, Jones WA (2006) Evaluation of *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycota: Hyphomycetes) strains isolated from *Varroa* mites in southern France. J Apic Res 45:219–220

Meikle WG, Mercadier G, Holst N, Nansen C, Girod V (2007) Duration and spread of an entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Viullemin (Deuteromycota: Hyphomycetes), used to treat *Varroa* mites, *Varroa destructor* Anderson and Trueman (Acari: Varroidae), in honeybee colonies. J Econ Entomol 100:1–10

Meikle WG, Mercadier G, Holst N, Nansen C, Girod V (2008) Impact of a treatment of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) on honeybee (Hymenoptera: Apidae) colony health and on *Varroa* mites (Acari: Varroidae). Apidologie. doi:10.1051/apido:20007057

Rinderer TE, de Guzman LI, Delatte GT, Stelzer JA, Lancaster VA, Kuznetsov V, Beaman L, Watts R, Harris JW (2001) Resistance to the parasitic mite *Varroa destructor* in honey bees from far-eastern Russia. Apidologie 32:381–394

Shaw KE, Davidson G, Clark SJ, Ball BV, Pell JK, Chandler D, Sunderland KD (2002) Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*. Biol Contr 24:266–276

Simpson J (1961) Nest climate regulation in honey bee colonies. Science 33:1327–1333

Southwick EE (1991) The colony as a thermoregulating superorganisms. In: Goodman LJ, Fisher RC (eds) The behavior and physiology of bees. CAB International, Wallingford, UK

Tanada Y, Kaya HK (1993) Insect pathology. Academic Press, San Diego, CA, USA

Winston ML (1987) The biology of the honey bee. Harvard Univ Press, Cambridge, MA, USA